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<b>(54) Title:</b> A NOVEL METHOD FOR IMPORTING BIOLOGICALLY ACTIVE MOLECULES INTO CELLS  <b>(57) Abstract</b>  The present invention relates to the delivery of biologically active molecules, such as peptides, nucleic acids and therapeutic agents, into the interior of cells by administering to the cells a complex comprising the molecule linked to an importation competent signal peptide. Such delivery can be utilized for purposes such as peptide therapy, gene transfer, and antisense therapy.		

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5                   **A NOVEL METHOD FOR IMPORTING BIOLOGICALLY  
                    ACTIVE MOLECULES INTO CELLS**

10                   **BACKGROUND OF THE INVENTION**

**FIELD OF THE INVENTION**

The present invention relates generally to the delivery of biologically active molecules, such as peptides, polypeptides and nucleic acids, into the interior of cells.

15   Specifically, the delivery is accomplished by administering to the cells a complex comprising the molecule linked to a signal peptide. Such delivery can be utilized for many purposes such as peptide therapy, antisense oligonucleotide therapy and gene transfer.

**BACKGROUND ART**

20                   Peptides have been developed for many therapeutic uses. For example, diseases currently targeted by new peptide drugs include heart conditions, cancers, endocrine disorders, neurological defects, respiratory conditions, allergies and autoimmune diseases. Although the manufacture of known therapeutic peptides can be achieved by known methods, i.e., classic synthetic techniques or recombinant genetic engineering,  
25   delivery of the peptides into a cell has remained problematic, since they cannot readily cross biological membranes to enter cells. Thus, current methods include permeabilization of the cell membrane or microinjection into the cell. Both of these methods have serious drawbacks. Permeabilization of cells, e.g., by saponin, bacterial toxins, calcium phosphate, electroporation, etc., can only be practically useful for *ex vivo* methods, and these methods  
30   cause damage to the cells. Microinjection requires highly skilled technicians (thus limiting

its use to a laboratory setting), it physically damages the cells, and it has only limited applications as it cannot be used to treat, for example, a mass of cells or an entire tissue, because one cannot feasibly inject large numbers of cells.

5                   Similarly, delivery of nucleic acids has been problematic. Methods currently employed include the permeabilization described above, with the above-described drawbacks, as well as vector-based delivery, such as with viral vectors, and liposome-mediated delivery. However, viral vectors can present additional risks to a patient, and liposome techniques have not achieved satisfactorily high levels of delivery into cells.

10

                  Signal peptide sequences,<sup>1</sup> which share the common motif of hydrophobicity, mediate translocation of most intracellular secretory proteins across mammalian endoplasmic reticulum (ER) and prokaryotic plasma membranes through the putative protein-conducting channels.<sup>2-11</sup> Alternative models for secretory protein transport also  
15 support a role for the signal sequence in targeting proteins to membranes.<sup>12-15</sup>

                  Several types of signal sequence-mediated inside-out membrane translocation pathways have been proposed. The major model implies that the proteins are transported across membranes through a hydrophilic protein-conducting channel formed by  
20 a number of membrane proteins.<sup>2-11</sup> In eukaryotes, newly synthesized proteins in the cytoplasm are targeted to the ER membrane by signal sequences that are recognized generally by the signal recognition particle (SRP) and its ER membrane receptors. This targeting step is followed by the actual transfer of protein across the ER membrane and out of the cell through the putative protein-conducting channel (for recent reviews,  
25 see references 2-5). In bacteria, the transport of most proteins across the cytoplasmic membrane also requires a similar protein-conducting channel.<sup>7-11</sup> On the other hand, signal peptides can interact strongly with lipids, supporting the proposal that the transport of some secretory proteins across cellular membranes may occur directly through the lipid bilayer in the absence of any proteinaceous channels.<sup>14-15</sup>

Thus, though many attempts have been made to develop effective methods for importing biologically active molecules into cells, both *in vivo* and *in vitro*, none has proved to be entirely satisfactory. This problem affects a wide variety of therapies. The solution of this problem would greatly expand treatments of diseases for which delivery of a therapeutic molecule would be beneficial. The present invention solves this long-felt, broad spectrum problem by providing a method of importing a biologically active molecule into a cell using mechanisms naturally occurring in cells and thus avoiding damaging the target cells. Additionally, the present method can be used to import molecules into large numbers of cells, including organs. Thus, this versatile inventive method can be employed in numerous treatments of diseases and disorders.

#### SUMMARY OF THE INVENTION

The present invention provides a method of importing a biologically active molecule into a cell *ex vivo* comprising administering to the cell, under import conditions, a complex comprising the molecule linked to an importation competent signal peptide, thereby importing the molecule into the cell. Molecules that can be delivered by this method can include, for example, peptides, polypeptides, proteins, nucleic acids, carbohydrates, lipids, glycolipids, and therapeutic agents.

20

The present invention further provides a method of importing a biologically active molecule into a cell in a subject comprising administering to the subject a complex comprising the molecule linked to an importation competent signal peptide, thereby importing the molecule into the cell of the subject.

25

Additionally, the instant invention provides a method of importing a biologically active molecule into the nucleus of a cell in a subject comprising administering to the subject a complex comprising the molecule linked to an importation competent signal

peptide and a nuclear localization peptide, thereby importing the molecule into the nucleus of the cell of the subject.

Further, the present invention provides a method of regulating growth of a  
5 cell in a subject comprising administering to the subject a complex comprising a growth regulatory peptide linked to an importation competent signal peptide to import the growth regulatory peptide into the cell of the subject, thereby regulating the growth of the cell.

The present invention additionally provides a method of inhibiting expression  
10 in a cell in a subject of a gene controlled by transcription factor NF- $\kappa$ B comprising administering to the subject a complex comprising an importation competent signal peptide linked to a nuclear localization peptide of an active subunit of NF- $\kappa$ B complex.

Also, the present invention provides a method of stimulating the immune  
15 system of a subject comprising administering to the subject a complex comprising an importation competent signal peptide linked to an antigenic peptide.

The instant invention also provides a complex comprising a biologically  
active molecule linked to an importation competent signal peptide and to a nuclear  
20 localization peptide.

The present invention further provides a complex comprising an importation  
competent signal peptide linked to a biologically active molecule selected from the group  
consisting of a nucleic acid, a carbohydrate, a lipid, a glycolipid and a therapeutic agent.  
25

The present invention also provides a method of screening signal peptides  
for the ability to effect the importation of a biologically active molecule into a cell  
comprising administering to the cell a complex comprising the molecule linked to the signal

peptide and determining whether the molecule is imported into the cell, the presence of importation of the molecule indicating a signal peptide which can effect importation.

### BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1 is a graphic representation of [ $^3\text{H}$ ] thymidine incorporation by NIH 3T3 cells stimulated with either (a) SA peptide, SA $\alpha$  peptide, ANL peptide or SM peptide or (b) acidic Fibroblast Growth Factor (aFGF).

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### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the Examples and Figures included therein.

15

The present invention provides the discovery that importing exogenous biologically active molecules into intact cells can be engineered by forming a complex by attaching an importation competent signal peptide sequence to a selected biologically active molecule and administering the complex to the cell. The complex is then imported across the cell membrane by the cell. Thus, the present invention provides a method of importing a biologically active molecule into a cell *ex vivo* or *in vivo* comprising administering to the cell, under import conditions, a complex comprising the molecule linked to an importation competent signal peptide, thereby importing the molecule into the cell.

25

As used herein, "biologically active molecule" includes any molecule which, if imported into a cell, can have a biological effect. Naturally only those molecules which are of a size which can be imported into the cell are within the scope of the invention. However, since very large proteins (ranging from molecular weights of about 100,000 to around 1 million) are exported by cells (e.g., antibodies, fibrinogen, and macroglobulin),

very large proteins can be imported into cells by this method. Therefore, size ranges for proteins from a few amino acids to around a thousand amino acids can be used. A preferable size range for proteins is from a few amino acids to about 250 amino acids. For any molecule, size ranges can be up to about a molecular weight of about 1 million, with a  
5 preferable size range being up to a molecular weight of about 25,000, and an even more preferable size range being up to a molecular weight of about 3,000. In addition, only those molecules which can be linked to a signal peptide, either directly or indirectly, are within the scope of the invention. Likewise, the present invention requires that the complex is administered under suitable conditions for effective import into the cell.

10

Examples of biologically active molecules include proteins, polypeptides and peptides, which include functional domains of biologically active molecules, such as growth factors, enzymes, transcription factors, toxins, antigenic peptides (as for vaccines), antibodies, and antibody fragments. Additional examples of biologically active molecules  
15 include nucleic acids, such as plasmids, coding DNA sequences, mRNAs and antisense RNA molecules, carbohydrates, lipids and glycolipids. Further examples of biologically active molecules include therapeutic agents, in particular those with a low cell membrane permeability. Some examples of these therapeutic agents include cancer drugs, such as Daunorubicin,<sup>26</sup> and toxic chemicals which, because of the lower dosage that can be  
20 administered by this method, can now be more safely administered.

A specific example of a biologically active molecule is the peptide comprising the nuclear localization sequence (NLS) of acidic fibroblast growth factor (aFGF), listed herein as SEQ ID NO:2. As demonstrated in the examples below, the NLS  
25 of aFGF, when linked to a signal peptide and transported into cells (e.g., the entire peptide listed herein as SEQ ID NO:4), induces a mitogenic response in the cells. Another example of a biologically active molecule is the peptide comprising the NLS of transcription factor NF- $\kappa$ B subunit p50, listed herein as SEQ ID NO:10. As shown in the examples herein, when a peptide comprising the signal sequence of K-FGF and the NLS of transcription



factor NF- $\kappa$ B p50 subunit, this peptide (called SN50) being listed herein as SEQ ID NO:9, is transfected into cells having transcription factor NF- $\kappa$ B, the normal translocation of active NF- $\kappa$ B complex into the nucleus is inhibited. In this manner, cell growth can be inhibited by inhibiting the action of NF- $\kappa$ B and therefore inhibiting the expression of genes controlled by transcription factor NF- $\kappa$ B.

Yet another example of a biologically active molecule is an antigenic peptide. Antigenic peptides can be administered to provide immunological protection when imported by cells involved in the immune response. Other examples include immunosuppressive peptides (e.g., peptides that block autoreactive T cells, which peptides are known in the art). Numerous other examples will be apparent to the skilled artisan.

Suitable import conditions are exemplified herein and include cell and complex temperature between about 18°C and about 42°C, with a preferred temperature being between about 22°C and about 37°C. For administration to a cell in a subject, the complex, once in the subject, will of course adjust to the subject's body temperature. For *ex vivo* administration, the complex can be administered by any standard methods that would maintain viability of the cells, such as by adding it to culture medium (appropriate for the target cells) and adding this medium directly to the cells. As is known in the art, any medium used in this method can be aqueous and non-toxic so as not to render the cells non-viable. In addition, it can contain standard nutrients for maintaining viability of cells, if desired. For *in vivo* administration, the complex can be added to, for example, a blood sample or a tissue sample from the patient, or to a pharmaceutically acceptable carrier, e.g., saline and buffered saline, and administered by any of several means known in the art. Examples of administration include parenteral administration, e.g., by intravenous injection including regional perfusion through a blood vessel supplying the tissues(s) or organ(s) having the target cell(s), or by inhalation of an aerosol, subcutaneous or intramuscular injection, topical administration such as to skin wounds and lesions, direct transfection into, e.g., bone marrow cells prepared for transplantation and subsequent transplantation into the

subject, and direct transfection into an organ that is subsequently transplanted into the subject. Further administration methods include oral administration, particularly when the complex is encapsulated, or rectal administration, particularly when the complex is in suppository form. A pharmaceutically acceptable carrier includes any material that is not  
5 biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected complex without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

10 Administration can be performed for a time length of about 1 minute to about 72 hours. Preferable time lengths are about 5 minutes to about 48 hours, and even more preferably about 5 minutes to about 20 hours, and even more preferably about 5 minutes to about 2 hours. Optimal time lengths and conditions for any specific complex and any specific target cell can readily be determined, given the teachings herein and knowledge  
15 in the art.<sup>27</sup> Specifically, if a particular cell type *in vivo* is to be targeted, for example, by regional perfusion of an organ or tumor, cells from the target tissue can be biopsied and optimal dosages for import of the complex into that tissue can be determined *in vitro*, as described herein and as known in the art, to optimize the *in vivo* dosage, including concentration and time length. Alternatively, culture cells of the same cell type can also be  
20 used to optimize the dosage for the target cells *in vivo*.

For either *ex vivo* or *in vivo* use, the complex can be administered at any effective concentration. An effective concentration is that amount that results in importation of the biologically active molecule into the cell. Such a concentration will  
25 typically be between about 0.5 nM to about 100  $\mu$ M (culture medium concentration (*ex vivo*) or blood serum concentration (*in vivo*)). Optimal concentrations for a particular complex and/or a particular target cell can be readily determined following the teachings herein. Thus, *in vivo* dosages of the complex include those which will cause the blood serum concentration of the complex to be about 0.5 nM to about 100  $\mu$ M. A preferable

concentration is about 2 nM to about 50  $\mu$ M. The amount of the complex administered will, of course, depend upon the subject being treated, the subject's age and weight, the manner of administration, and the judgment of the skilled administrator. The exact amount of the complex will further depend upon the general condition of the subject, the severity of the disease/condition being treated by the administration and the particular complex chosen. However, an appropriate amount can be determined by one of ordinary skill in the art using routine optimization given the teachings herein.

Parenteral administration, e.g., regional perfusion, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, such as liquid solutions, suspensions, or emulsions. A slow release or sustained release system, such as disclosed in U.S. Patent No. 3,710,795, can also be used, allowing the maintenance of a constant level of dosage.

Depending on the intended mode of administration, the pharmaceutical compositions may be in the form of solid, semi-solid or liquid dosage forms, such as, for example, tablets, suppositories, pills, capsules, powders, liquids, suspensions, lotions, creams, gels, or the like, preferably in unit dosage form suitable for single administration of a precise dosage. The compositions will include, as noted above, an effective amount of the selected drug in combination with a pharmaceutically acceptable carrier and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc.

For solid compositions, conventional nontoxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talc, cellulose, glucose, sucrose, magnesium carbonate, and the like. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc. an active compound as described herein and optional pharmaceutical adjuvants in an excipient, such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the

pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington's Pharmaceutical Sciences*.<sup>27</sup>

5

The present invention utilizes a complex comprising the selected biologically active molecule linked to an importation competent signal peptide. As discussed above, the biologically active molecule can be selected from any of a variety of molecules, with its selection being dependent upon the purpose to be accomplished by importing the molecule

10 into the selected cell. An "importation competent signal peptide," as used herein, is a sequence of amino acids generally of a length of about 10 to about 50 or more amino acid residues, many (typically about 55-60%) residues of which are hydrophobic such that they have a hydrophobic, lipid-soluble portion.<sup>1</sup> The hydrophobic portion is a common, major motif of the signal peptide, and it is often a central part of the signal peptide of protein

15 secreted from cells. A signal peptide is a peptide capable of penetrating through the cell membrane to allow the export of cellular proteins. The signal peptides of this invention, as discovered herein, are also "importation competent," i.e., capable of penetrating through the cell membrane from outside the cell to the interior of the cell. The amino acid residues can be mutated and/or modified (i.e., to form mimetics) so long as the modifications do not

20 affect the translocation-mediating function of the peptide. Thus the word "peptide" includes mimetics and the word "amino acid" includes modified amino acids, as used herein, unusual amino acids, and D-form amino acids. All importation competent signal peptides encompassed by this invention have the function of mediating translocation across a cell membrane from outside the cell to the interior of the cell. Such importation competent

25 signal peptides could potentially be modified such that they lose the ability to export a protein but maintain the ability to import molecules into the cell. A putative signal peptide can easily be tested for this importation activity following the teachings provided herein, including testing for specificity for any selected cell type.

Signal peptides can be selected, for example, from the SIGPEP database, which also lists the origin of the signal peptide.<sup>30, 38</sup> When a specific cell type is to be targeted, a signal peptide used by that cell type can be chosen. For example, signal peptides encoded by a particular oncogene can be selected for use in targeting cells in which the oncogene is expressed. Additionally, signal peptides endogenous to the cell type can be chosen for importing biologically active molecules into that cell type. And again, any selected signal peptide can be routinely tested for the ability to translocate across the cell membrane of any given cell type according to the teachings herein. Specifically, the signal peptide of choice can be conjugated to a biologically active molecule, e.g., a functional domain of a cellular protein or a reporter construct, and administered to a cell, and the cell is subsequently screened for the presence of the active molecule.

The presence of modified amino acids in the signal peptide can additionally be useful for rendering a complex, wherein the biologically active molecule is a peptide, polypeptide or protein, more resistant to peptidases in the subject. Thus these signal peptides can allow for more effective treatment by allowing more peptides to reach their target and by prolonging the life of the peptide before it is degraded. Additionally, one can modify the amino acid sequence of the signal peptide to alter any proteolytic cleavage site present in the original signal sequence for removing the signal sequence. Clearance sites are characterized by small, positively charged amino acids with no side chains and are localized within about 1 to about 4 amino acids from the carboxy end of the signal peptide.<sup>1</sup>

An example of a useful signal peptide is the signal peptide from Kaposi fibroblast growth factor (K-FGF),<sup>16-17</sup> listed herein as SEQ ID NO:5. Any signal peptide, however, capable of translocating across the cell membrane into the interior of the selected target cell can be used according to this invention.

By "linked" as used herein is meant that the biologically active molecule is associated with the signal peptide in such a manner that when the signal peptide crosses the

cell membrane, the molecule is also imported across the cell membrane. Examples of such means of linking include (1) when the molecule is a peptide, the signal peptide (and a nuclear localization peptide, if desired) can be linked by a peptide bond, i.e., the two peptides can be synthesized contiguously; (2) when the molecule is a polypeptide or a protein (including antibody), the signal peptide (and a nuclear localization peptide, if desired) can be linked to the molecule by a peptide bond or by a non-peptide covalent bond (such as conjugating a signal peptide to a protein with a crosslinking reagent); (3) for molecules that have a negative charge, such as nucleic acids, the molecule and the signal peptide (and a nuclear localization peptide, if desired) can be joined by charge-association between the negatively-charged molecule and the positively-charged amino acids in the peptide or by other types of association between nucleic acids and amino acids; (4) chemical ligation methods can be employed to create a covalent bond between the carboxy-terminal amino acid of the signal peptide (and a nuclear localization peptide, if desired) and the molecule. Methods (1) and (2) are typically preferred.

15

Examples of method (1) are shown below wherein a peptide is synthesized, by standard means known in the art,<sup>24,25</sup> that contains, in linear order from the amino-terminal end, a signal peptide sequence, an optional spacer amino acid region, and a biologically active amino acid sequence. Such a peptide could also be produced through recombinant DNA techniques, expressed from a recombinant construct encoding the above-described amino acids to create the peptide.<sup>28</sup>

For method (2), either a peptide bond, as above, can be utilized or a non-peptide covalent bond can be used to link the signal peptide with the biologically active polypeptide or protein. This non-peptide covalent bond can be formed by methods standard in the art, such as by conjugating the signal peptide to the polypeptide or protein via a crosslinking reagent, for example, glutaraldehyde. Such methods are standard in the art.<sup>29</sup> For method (3) the molecules can simply be mixed with the signal peptide and thus allowed to associate. These methods are performed in the same manner as association of nucleic

acids with cationic liposomes.<sup>32-34</sup> Alternatively, covalent (thioester) bonds can be formed between nucleic acids and peptides. Such methods are standard in the art.

For method (4), standard chemical ligation methods, such as using chemical crosslinkers interacting with the carboxy-terminal amino acid of the signal peptide, can be utilized. Such methods are standard in the art (*see*, e.g., Goodfriend,<sup>31</sup> which uses water-soluble carbodiimide as a ligating reagent) and can readily be performed to link the carboxy terminal end of the signal peptide to any selected biologically active molecule.

The complex that is administered to a subject can further comprise a liposome. Cationic and anionic liposomes are contemplated by this invention, as well as liposomes having neutral lipids. Cationic liposomes can be complexed with the signal peptide and a negatively-charged biologically active molecule by mixing these components and allowing them to charge-associate. Cationic liposomes are particularly useful when the biologically active molecule is a nucleic acid because of the nucleic acid's negative charge. Examples of cationic liposomes include lipofectin, lipofectamine, lipofectace and DOTAP.<sup>32-34</sup> Anionic liposomes generally are utilized to encase within the liposome the substances to be delivered to the cell. Procedures for forming cationic liposomes encasing substances are standard in the art<sup>35</sup> and can readily be utilized herein by one of ordinary skill in the art to encase the complex of this invention.

Any selected cell into which import of a biologically active molecule would be useful can be targeted by this method, as long as there is a means to bring the complex in contact with the selected cell. Cells can be within a tissue or organ, for example, supplied by a blood vessel into which the complex is administered. Additionally, the cell can be targeted by, for example, inhalation of the molecule linked to the peptide to target the lung epithelium. Some examples of cells that can be targeted by this inventive method include fibroblasts, epithelial cells, endothelial cells, blood cells and tumor cells, among many. In addition, the complex can be administered directly to a tissue site in the body. As discussed

above, the signal peptide utilized can be chosen from signal peptides known to be utilized by the selected target cell, or a desired signal peptide can be tested for importing ability given the teachings herein. Generally, however, all signal peptides have the common ability to cross cell membranes due, at least in part, to their hydrophobic character. Thus, in  
5 general, a membrane-permeable signal peptide can be designed and used for any cell type, since all eukaryotic cell membranes have a similar lipid bilayer.

One particularly useful example is to import an antigenic peptide into cells of the immune system, thereby allowing the antigen to be presented by antigen-presenting  
10 cells, and an immune response to the antigen to be developed by the subject. These antigenic peptide-containing complexes can be administered to the subject according to standard methods of administering vaccines, e.g., intramuscularly, subcutaneously or orally, and effectiveness can be measured by subsequent measuring of the presence of antibodies to the antigen.

15

The present invention also provides a method of importing a biologically active molecule into the nucleus of a cell in a subject comprising administering to the subject a complex comprising the molecule linked to an importation competent signal peptide and a nuclear localization peptide, thereby importing the molecule into the nucleus  
20 of the cell of the subject. A nuclear localization peptide, as used herein, is a peptide having the function of delivering an intracellular peptide into the nucleus of the cell. Such nuclear localization sequences are known in the art to have this function.<sup>36,37</sup> An example of a nuclear localization peptide is the nuclear localization sequence of aFGF, listed herein as SEQ ID NO:2. An example of a signal peptide (K-FGF) linked to a nuclear localization  
25 peptide (aFGF) is set forth in SEQ ID NO:3. As these examples demonstrate, the nuclear localization peptide sequences can be synthesized as a peptide contiguous with the signal peptide, if desired. Additionally, separate peptides can be linked by any means such as described herein.



The present invention also provides a method of regulating growth of a cell in a subject comprising administering to the subject a complex comprising a growth regulatory peptide linked to an importation competent signal peptide to import the growth regulatory peptide into the cell of the subject, thereby regulating the growth of the cell.

- 5 Growth can be stimulated or inhibited depending upon the growth regulatory peptide selected. It is to be noted that the present invention provides regulation of cell growth also by administering a nucleic acid encoding a growth regulatory peptide under functional control of a suitable promoter for expression in a specific target cell, wherein the nucleic acid is complexed with a signal peptide and administered to the target cell.

10

- There are numerous growth regulatory peptides known in the art, any of which can be utilized in this invention, if appropriate for the target cell type and the type of regulation desired. The signal peptide facilitates the efficient import of the growth regulatory peptide into the target cell and, once the regulatory peptide is imported, it
- 15 functions to regulate cell growth in its specific manner. A particularly useful target cell is a tumor cell in which the method can be used to inhibit further aberrant cell growth. Cell growth can be stimulated by administering a growth regulatory peptide comprising the nuclear localization sequence of acidic fibroblast growth factor (aFGF). Cell growth can be inhibited by administering peptides that inhibit growth, for example peptides that inhibit
- 20 transcription in the cell, such as the NLS of the p50 subunit of transcription factor NF- $\kappa$ B.

- An example of this method is seen below in the examples wherein the growth regulatory peptide stimulates cell growth and comprises the nuclear localization signal of aFGF. As this example demonstrates, the growth regulatory peptide, if desired,
- 25 can be synthesized contiguously with the signal peptide, though any known method can be utilized to link them. An example of a contiguous peptide is set forth in SEQ ID NO:3 and SEQ ID NO:4. Another example is provided below, wherein a complex (listed as SEQ ID NO:9) comprising the membrane-permeable signal peptide of K-FGF linked to the NLS of

transcription factor NF- $\kappa$ B p50 subunit is administered and inhibits the expression of genes encoding pro-inflammatory mediators.

The invention also provides a method of inhibiting expression in a cell in a  
5 subject of a gene controlled by transcription factor NF- $\kappa$ B comprising administering to the  
subject a complex comprising an importation competent signal peptide linked to a nuclear  
localization peptide of an active subunit of NF- $\kappa$ B complex. Many genes controlled by NF-  
 $\kappa$ B are known in the art, and others can be readily tested by standard means. Examples of  
such genes include cytokines and interleukins, such as IL-1, IL-6, granular colony  
10 stimulating factor, plasminogen activator inhibitor and procoagulant tissue factor.  
Additionally, organisms having genes affected by NF- $\kappa$ B can be inhibited by this method,  
such organisms including human immunodeficiency virus (HIV) and cytomegalovirus  
(CMV). The optimal inhibitory peptide for specific cell types and specific genes can readily  
be determined by standard methods given the teachings herein. Additionally, the optimal  
15 inhibitory peptide for a specific cell type subjected to a specific stimulant can readily be  
determined.

An example is provided herein wherein translocation of the NF- $\kappa$ B complex  
to the nucleus in endothelial cells stimulated with lipopolysaccharide (LPS) is inhibited by a  
20 complex comprising a signal peptide linked to the NLS of subunit p50 of NF- $\kappa$ B.  
Presumably, the NLS of subunit p50 interferes with translocation of the complex to the  
nucleus due to competitive binding. Any cell type subjected to any (or no) stimulus can be  
readily screened for the optimal inhibitory peptide, i.e., the optimal NLS of a subunit of NF-  
 $\kappa$ B, for that cell type. For example, for LEII cells, as demonstrated herein, the NLS of p50  
25 is optimal.

The subunits of NF- $\kappa$ B complex are known in the art.<sup>43</sup> They include p50,  
p65 and cellular REL (c-REL). The nuclear localization sequences of these subunits are  
also known. An "active" subunit of NF- $\kappa$ B complex, as used herein, means a subunit which,

when it is inhibited, causes transcription factor NF- $\kappa$ B not to function to mediate transcription of genes under its control. The nuclear localization peptide used in this method can be a modification of the known NLS of these subunits as long as it retains the function of inhibiting expression of a gene controlled by NF- $\kappa$ B, as can be readily  
5 determined according to the teachings herein and knowledge in the art.

The invention further provides a method of stimulating the immune system of a subject comprising administering to the subject a complex comprising an importation competent signal peptide linked to an antigenic peptide. The complex can be administered  
10 to the subject by standard means known in the art for administering vaccines. The method can facilitate uptake of the antigen into cells for subsequent antigen presentation and the resultant known cascade of the immune system to result in the stimulation of immunity to the antigen.

15 Furthermore, if known peptides for blocking autoreactive T cells are linked to a signal peptide and administered to a subject, an immunosuppressive effect can be stimulated in the subject. Such a method of stimulating immunosuppression can be used to treat autoimmune diseases such as multiple sclerosis. These blocking peptides can also be administered by known methods for administering peptides, such as methods for  
20 administering vaccines.

The invention also provides a complex comprising a biologically active molecule linked to an importation competent signal peptide and to a nuclear localization peptide. The linkage can be made as described above or otherwise known in the art.  
25 Though, as described above, any signal peptide and any nuclear localization sequence can be utilized, such a complex is exemplified by the amino acid sequences set forth in SEQ ID NO:3 and SEQ ID NO:4, which contain the K-FGF signal peptide (SEQ ID NO:5) linked to the aFGF nuclear localization peptide (SEQ ID NO:2).

The invention further provides a complex comprising an importation competent signal peptide linked to biologically active molecule selected from the group consisting of a nucleic acid, a carbohydrate, a lipid, a glycolipid and a therapeutic agent. This complex can further comprise a liposome. These complexes can be formed as described above. Liposomes can be selected as described above. The complex can be placed in a pharmaceutically acceptable carrier.

As used herein, "a" can mean one or more, depending on the context in which it is used.

The invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

#### 15 Statement Concerning Utility

The present method, which provides an effective method for importing biologically active molecules into cells, has many uses, both *in vivo* and *ex vivo*. Specific utilities using the method are apparent and are exemplified as follows. *In vivo*, the method can be used to deliver into cells therapeutic molecules, such as peptides and proteins to regulate aberrant functions or to supply deficient cells; DNA for gene therapy (e.g., to provide the CFTR gene in cystic fibrosis patients); RNA for antisense therapy (e.g., to inhibit growth as in inhibiting expression in cancer cells); and therapeutic agents such as cancer drugs or toxic chemicals (which can be administered in lower dosages with this method as compared to previous methods not utilizing a signal peptide to more efficiently enter the cells). *Ex vivo*, the method allows efficient transfection of cells without performing cell-damaging procedures. Therefore, this method is useful *ex vivo* in any method that utilizes transfection, such as transfecting reporter genes into cells to screen for compounds that affect expression of the reporter gene, and for transfecting bone marrow

cells, blood cells, cells of an organ for subsequent transplantation into a subject, or culture cells, with a gene to effect protein expression in the cells.

More specifically, this method can be used for anti-thrombotic therapy by  
5 administering functional domains of known cell receptors which mediate aggregation of platelets, by competitive binding. Additionally, the method can be used for immunosuppression in autoimmune diseases by introducing immunosuppressive peptides into cells involved in the immune response. Furthermore, growth inhibitors can be administered by this method to tumor cells to treat, for example, cancer cells.

10

This method can also be used to facilitate the absorption of biologically active molecules from, e.g., the mouth, stomach or intestinal tract by facilitating movement of the molecules into the connective tissue beneath the lining of the digestive tract. Also, by allowing one to design signal peptides with modified amino acids, one can stabilize  
15 biologically active peptides by making them more resistant to peptidases and therefore also prolong the action of the peptide.

### EXAMPLES

20 The peptides used herein were synthesized by a stepwise solid-phase peptide synthesis approach<sup>24</sup> and purified by high performance liquid chromatography using C<sub>18</sub> reverse phase column as described.<sup>25</sup> The exact molecular weights of the purified peptides were confirmed by mass spectrometry analysis.

25 Amino acid residues 1-16 of the SM peptide were patterned after the predicted signal peptide sequence of K-FGF<sup>16,17</sup> (listed separately herein as SEQ ID NO:5), residues 17-19 were designed as a spacer, and residues 20-26 contain an epitope tag recognized by antibody (see SEQ ID NO:1). Amino acid residues 1-19 of the SA peptide are identical to those of the SM peptide. However, its carboxyl terminal residues 20-26 are

same as the sequence of the ANL peptide (SEQ ID NO:2), which is derived from the nuclear localization sequence of acidic FGF.<sup>18</sup> The SA peptide is listed as SEQ ID NO:3. The amino acid sequence of the SA $\alpha$  peptide was the same as that of the SA peptide except it had a two amino acid residue extension (Met-Pro) at the carboxyl terminus, which created an epitope (Leu-Met-Pro) for anti-SM peptide antibody. The SA $\alpha$  peptide is listed herein as SEQ ID NO:4.

#### Membrane-Permeable Signal Sequence Peptide (SM Peptide)

A 26-residue peptide (referred to as SM, listed herein as SEQ ID NO:1) that contained the predicted signal sequence of Kaposi fibroblast growth factor<sup>16,17</sup> (K-FGF) was chemically synthesized. An indirect immunofluorescence assay using antibody against epitope tag-containing SM peptide was employed to follow translocation of the SM peptide to the intracellular compartments of NIH 3T3 cells. A polyclonal anti-SM peptide antibody against the SM peptide-keyhole limpet haemocyanin conjugate (Pierce) was raised in rabbits and reacted with SM peptide in ELISA (titer > 1:30,000). The intracellular SM peptide was detected by an indirect immunofluorescence assay using affinity-purified anti-SM peptide IgG and rhodamine-labelled anti-rabbit antibody (Kirkegaard & Perry). Briefly, confluent NIH 3T3 cells on the chamber slides (Nunc) were treated with either 0.5 ml SM peptide solution (100  $\mu\text{g ml}^{-1}$ ) in DMEM containing 10% FBS or with 0.5 ml DMEM containing 10% FBS only for 30 minutes at 37°C. The cells were fixed with 3.5% paraformaldehyde solution in PBS followed by 0.25% Triton X-100 in PBS and then treated with 1:20 anti-SM peptide IgG in PBS containing 0.5% bovine serum albumin (BSA) for 1.5 h. The intracellular SM peptide-antibody complexes were visualized by subsequent incubation with a rhodamine-labelled anti-rabbit polyclonal antibody for 1 h. In control systems, anti-SM peptide antibody was preabsorbed with the SM peptide. Intracellular localization of the SA $\alpha$  peptide was detected by immunofluorescence assay using affinity-purified anti-SM peptide IgG as described above for SM peptide. Following incubation of cells with SM peptide, intracellular deposits were observed in almost all cells. A ten-step z-position sectional scanning of the SM peptide-treated cells by the confocal

laser scanning microscopy (CLSM) affirmed that these deposits were intracellular. Immunodetection of the SM peptide was specific because cells incubated with the peptide-antibody complex showed no evidence of intracellular peptide. Likewise, cells not exposed to SM peptide or cells exposed to SM peptide followed by the secondary antibody alone  
5 were negative. If cells were fixed with paraformaldehyde before peptide treatment, the cellular import of SM peptide was prevented.

To determine the rate of SM peptide import across cell membranes, a kinetic experiment was carried out with the SM peptide-treated NIH 3T3 cells. NIH 3T3 cells  
10 were treated at 37°C with 0.5 ml SM peptide at 100  $\mu\text{g mL}^{-1}$  in DMEM containing 10% FBS for 1, 5, 15, 30, 60 and 120 minutes. Intracellular SM peptide deposits were detected by indirect immunofluorescence assay as described above. The intracellular staining of intracellular SM peptide was observed during the first 5 minute interval and plateaued at about 30 minutes, indicating that the signal sequence-mediated peptide import into cells is  
15 rapid. The 30-minute time point was therefore selected to determine the optimal peptide concentrations for detectable import.

To determine the optimal peptide concentration, NIH 3T3 cells were treated for 30 minutes at 37°C with 0.5 ml SM peptide solution in DMEM containing 10% FBS at  
20 the following concentrations: 0, 2, 10, 50, 100, and 150  $\mu\text{g mL}^{-1}$ . Intracellular localization of the SM peptide was detected by indirect immunofluorescence assay as described above. Indirect immunofluorescence assay demonstrated detectable peptide in the form of intracellular punctate deposits when the cells were exposed to 2  $\mu\text{g/mL}$  (about 800 nM) of peptide. The cellular import was concentration-dependent and reached a plateau between 50  
25  $\mu\text{g/mL}$  and 100  $\mu\text{g/mL}$ .

Transport of the SM peptide across the cell membrane was temperature-dependent. No immunofluorescence staining was observed when the cells were treated for 30 minutes with 100  $\mu\text{g/mL}$  of the peptide at 4°C, whereas, cells treated at either 22°C or

37°C showed numerous punctate deposits. Accordingly, cellular import of the SM peptide resumed when the incubation temperature shifted from 4°C to 37°C.

Moreover, this signal sequence-mediated import is not limited to NIH 3T3 cells. The intracellular localization of SM peptide has been observed in baby hamster kidney-21 cells, human umbilical vein endothelial cells (HUVECs) and rodent endothelial cell line (LE-II), by the above indirect immunofluorescence assay, with the same results as with NIH 3T3 cells.

10 Membrane-Permeable Signal Peptide (SKP peptide)

Intracellular localization of the membrane-permeable peptide was also shown by treatment of cells with proteases following peptide import. For this experiment, 41-residue peptide (referred to as SKP and listed herein as SEQ ID NO:6) that contained the same hydrophobic sequence as SM peptide followed by the sequence of K-FGF(129-153) was designed and synthesized. The latter was present in KP peptide not containing hydrophobic sequence that served as the control for the membrane-permeable SKP peptide. Both peptides possess tyrosine residues, therefore they were radiolabeled with <sup>125</sup>I and examined for their ability to translocate into intact NIH 3T3 cells, as described below. Substantial radioactivity was detected in the <sup>125</sup>I-SKP peptide-treated cells but not in <sup>125</sup>I-KP peptide-treated cells, indicating that the import of SKP peptide into cells was selectively achieved due to the presence of hydrophobic, membrane-permeable sequence. The intracellular <sup>125</sup>I-SKP peptide was resistant to the action of proteases. After treatment of cells containing <sup>125</sup>I-SKP peptide with pronase or trypsin, no significant loss of cell-associated radioactivity was observed, suggesting that the <sup>125</sup>I-SKP peptide was located in intracellular compartments (Table 1). The import of membrane-permeable peptide was not dependent on ATP as high energy source because cells depleted of about 95% of ATP showed a similar <sup>125</sup>I-SKP peptide uptake as compared to ATP-positive cells (Table 1).



Both SKP and KP peptides were radiolabeled with  $^{125}\text{I}$  by the Iodogen method (Pierce). The specific activities of both peptides were similar ( $2.5 \times 10^4$  cpm/ng). NIH 3T3 cells were subcultured on a 60-mm dish and incubated at  $37^\circ\text{C}$  for 3 days. The confluent monolayers ( $1.6 \times 10^6$  cells) on each dish were then washed twice with PBS and

5 treated with 15 ng of  $^{125}\text{I}$ -SKP or  $^{125}\text{I}$ -KP peptide at  $37^\circ\text{C}$  for the indicated time. The cells were washed eight times with PBS and twice with 2 M NaCl buffer (pH 7.5) until no radioactivity could be detected in the washings. The washed cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, and 1% Triton X-100) and the radioactivity in the cell lysates was counted in

10 a Packard auto-gamma counter. In some experiments, the washed cells were further treated with pronase (1 mg/ml) or trypsin (0.05%) solution in DMEM for 5 min at  $37^\circ\text{C}$ . The supernatants and cells were separated and their radioactivities were counted separately. For ATP depletion assay, cells were incubated with 5  $\mu\text{g}/\text{ml}$  antimycin, 6.5 mM 2-deoxyglucose, and 10 mM glucono- $\delta$ -lactone in DMEM for 2 h at  $37^\circ\text{C}$  before addition of  $^{125}\text{I}$ -SKP

15 peptide. The levels of ATP in ATP-depleted cells and normal cells were determined by ATP bioluminescent assay kit (Sigma). No measurable ATP was observed in ATP-depleted cells.

TABLE 1

20 **IMPORT OF  $^{125}\text{I}$ -SKP PEPTIDE INTO ATP-DEPLETED NIH 3T3 CELLS AND EFFECT OF PROTEASES ON CELL-ASSOCIATED  $^{125}\text{I}$ -SKP PEPTIDE**

<u>ATP Depletion</u>		<u>Counts in cells (cpm/<math>1.6 \times 10^6</math> cells)</u>	
25	Untreated cells	$20,189 \pm 2,109$	
	APT-depleted cells	$22,266 \pm 3,602$	
<u>Protease Treatment</u>		<u>Counts in cell lysates</u>	<u>Counts in supernatants</u>
	Untreated cells	$21,323 \pm 853$	$2,966 \pm 838$
	Pronase	$21,791 \pm 1,953$	$1,979 \pm 75$
30	Trypsin	$23,193 \pm 310$	$655 \pm 70$

For ATP depletion assay, confluent NIH 3T3 cells ( $1.6 \times 10^6$  cells) in each dish were treated with or without ATP depleting reagents (antimycin, 2-deoxyglucose, and glucono- $\delta$ -lactone) for 2 h at 37°C. Cells were then treated with 15 ng of  $^{125}$ I-SKP peptide for 30 min at 37°C. After complete removal of extracellular  $^{125}$ I-labeled peptides, the radioactivity in the cell lysates was counted. For the assay using proteases, cells were treated with  $^{125}$ I-SKP peptide and washed as described above. The  $^{125}$ I-SKP peptide-associated cells were then treated with pronase (1 mg/ml), trypsin (0.05%), or diluent for 5 min at 37°C. The radioactivities in the cell lysates and supernatants were counted separately. Data in Table 1 represent the mean  $\pm$  SEM of triplicate determinations of a single experiment. The experiment was repeated three times with similar results.

#### Membrane-Permeable Signal Peptide with Functional Peptide Cargo (SA peptide)

Having demonstrated the feasibility of the cellular import of signal sequence-containing peptides, functional cargo in the form of a sequence responsible for the nuclear localization of cellular proteins was linked to a signal peptide. The nuclear localization sequence (NLS) of acidic FGF (aFGF), because it has previously been reported to play an essential role in aFGF mitogenic activity, was utilized.<sup>18</sup> It had previously been shown that a mutant aFGF with deletion in its NLS region Asn-Tyr-Lys-Lys-Pro-Lys-Leu (NYKKPKL), listed herein as SEQ ID NO:2, failed to stimulate DNA synthesis and cell proliferation *in vitro* although it could still bind to the FGF receptor and induce intracellular receptor-mediated tyrosine phosphorylation and c-fos expression.<sup>18</sup> Additionally, a recent study<sup>19</sup> of nuclear transport of aFGF suggested that translocation of aFGF to the nucleus was necessary for stimulating DNA synthesis by aFGF *in vitro*.

A 26-residue hybrid peptide (referred to as SA, listed herein as SEQ ID NO:3) was designed and synthesized. It contains the signal sequence of K-FGF<sup>16,17</sup> at its amino terminal region (residues 1-16 of SEQ ID NO:3) and a "functional cargo" in the form of a nuclear localization sequence (NLS) of aFGF<sup>18</sup> at its carboxyl terminal region (residues 20-26 of SEQ ID NO:3), separated by a spacer region of Ala-Ala-Ala (residues

17-19 of SEQ ID NO:3). Thus, the SA peptide differs from the SM peptide only in its 7-residue carboxyl terminal "cargo" region. A functional assay was performed in which SA peptide was able to induce a mitogenic response of NIH 3T3 cells measured by [<sup>3</sup>H]thymidine incorporation in a manner similar to aFGF bearing the same NLS.<sup>18</sup>

5

In this functional assay, confluent 3T3 cells grown initially in DMEM containing 10% FBS were transferred to a low serum medium (DMEM containing 0.5% FBS) for 2 days. The test peptides, either SA peptide, SA $\alpha$  peptide, ANL peptide, or SM peptide, or aFGF, were added to a fresh low serum medium at the indicated concentrations at 37°C. After 16 hours, [<sup>3</sup>H]thymidine was added and 4 hours later, the cells were washed with PBS, treated with trichloroacetic acid, solubilized with 0.15 M NaOH, and the radioactivity was determined in a liquid scintillation counter.

As shown in Fig. 1a, SA peptide stimulated [<sup>3</sup>H]thymidine incorporation 6-fold, while aFGF induced approximately an 8-fold stimulation in the same assay (Fig. 1b). Bars represent the mean  $\pm$  S.E.M. of at least three independent experiments done in triplicate and calculated as multiplicity of counts in the tested sample over the control sample. SA peptide within the concentration range used (0 to 46  $\mu$ M) was not cytotoxic as determined by staining with fluorescein diacetate/ethidium bromide.<sup>20</sup>

20

#### Mitogenic Activity of the SA Peptide

To determine whether the mitogenic activity of SA peptide required its full length sequence, two control peptides were examined in the same assay. They are the SM peptide containing signal peptide (listed herein as SEQ ID NO:1) and a 7-residue peptide (referred to as ANL, listed herein as SEQ ID NO:2) representing the NLS of aFGF. Neither control peptide showed any significant mitogenic activity when tested within comparable concentration ranges (Fig. 1a). These results suggest that neither the signal sequence alone (SM peptide) nor the nuclear localization sequence alone (ANL peptide) was sufficient for mitogenesis. SA peptide therefore was effective in mitogenesis because it

25

contained both the signal peptide sequence of K-FGF (for import into the cell) and nuclear localization sequence of aFGF (for mitogenic activity).

To further confirm the mitogenic activity of the SA peptide, its effect on  
5 DNA synthesis was examined. Serum-starved NIH 3T3 cells were treated with SA peptide, fixed, and the DNA concentration was determined by standard flow cytometric analysis. Specifically, confluent NIH 3T3 cells ( $1.3 \times 10^6$  cells) were serum-starved in DMEM containing 0.5% FBS for 2 days. The cells were untreated (control) or treated with SA peptide or aFGF for 20 h, harvested, spun down, and washed with serum-free PBS three  
10 times. The cells were fixed with methanol precooled to  $-20^\circ\text{C}$  for DNA analysis carried out by the Flow Cytometry Research Service of Vanderbilt University. The data were the mean  $\pm$  S.E.M. of six measurements and were analyzed for statistical significance by analysis of variance.

As shown in Table 2, the DNA synthesis in the S-phase of the cell cycle was  
15 significantly increased when the cells were treated with the SA peptide at  $100 \mu\text{g/ml}$ , which coincided with the active concentration in the thymidine incorporation assay (Fig. 1a). This result further confirms the role of the NLS region of aFGF in mitogenesis.<sup>18</sup> Thus, these data also are consistent with a recent demonstration using a genetic approach that schwannoma-derived growth factor requires NLS to exert its mitogenic activity.<sup>21</sup>

20

TABLE 2

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**DNA SYNTHESIS STIMULATED BY SA PEPTIDE  
AS COMPARED TO aFGF**

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	<u>Stimulus</u>	<u>Diploid % S Phase</u>
25	Control	$7.2 \pm 0.7$
	SA ( $50 \mu\text{g ml}^{-1}$ )	$6.7 \pm 0.9$
	SA ( $100 \mu\text{g ml}^{-1}$ )	$13.1 \pm 0.5$ ( $P < 0.05$ )
	aFGF ( $15 \text{ ng ml}^{-1}$ )	$27.8 \pm 2.3$ ( $P < 0.05$ )

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However, compared with aFGF, the SA peptide is less mitogenically potent in both thymidine incorporation and DNA analysis assays (Fig. 1 and Table 2). aFGF binds to the FGF receptors on NIH 3T3 cells and induces the tyrosine phosphorylation of a number of intracellular proteins that have been suggested as the FGF receptor signalling substrates.<sup>22,23</sup> In contrast, SA peptide did not stimulate the tyrosine phosphorylation of these intracellular proteins in the same cells even at the concentrations sufficient to induce DNA synthesis. Taken together, these results, make it unlikely that the mitogenic effect of SA peptide was mediated by FGF receptors.

#### 10 Immunofluorescence Assay for Modified SA Peptide

The intracellular SA peptide could not be tracked by an immunofluorescence assay because it was not recognized by the available anti-SM peptide antibody. However, attaching two extra amino acid residues (Met-Pro) to the carboxyl terminus of the SA peptide produced a modified SA peptide (referred to as SA $\alpha$ , listed herein as SEQ ID NO:4) that contained a 3-amino acid epitope tag, Leu-Met-Pro, recognized by anti-SM peptide antibody in ELISA. Accordingly, intracellular SA $\alpha$  peptide was observed in a punctate staining pattern in the SA $\alpha$ -treated NIH 3T3 cells by an indirect immunofluorescence assay using anti-SM peptide antibody. Like SA peptide, SA $\alpha$  peptide was mitogenic in the thymidine incorporation assay. These results are consistent with the relationship between the SA peptides' import into the intracellular compartments and their mitogenic activity.

#### Membrane-Permeable Signal Peptide with Functional Peptide Cargo (SN50)

Having demonstrated the feasibility of the cellular import of membrane-permeable SM and SKP peptides, another functional cargo was attached to the amino-terminal hydrophobic sequence conferring membrane-permeable capacity. For this purpose a sequence representing a functional domain of the nuclear factor  $\kappa$ B (NF- $\kappa$ B) responsible for a nuclear localization signal was selected. Import of such a peptide into the cell would be measured by inhibition of nuclear translocation of NF- $\kappa$ B complex in stimulated cells.

The NF- $\kappa$ B is a pleiotropic activator<sup>39,40</sup> that plays a critical role in the regulation of a number of cellular and viral genes, including the enhancer of human immunodeficiency virus (HIV). The inactive cytosolic form of NF- $\kappa$ B is a heterotrimer including p50, p65 and an inhibitory protein I $\kappa$ B.<sup>41,42</sup> Upon activation of cells with stimuli such as lipopolysaccharide (LPS) or cytokines,<sup>43,44,45</sup> I $\kappa$ B dissociates from the complex. This dissociation allows the translocation of heterodimer of p50 and p65 subunits to the nucleus. Both p50 and p65 subunits contain NLS, suggesting that the NLS sequence may be important for nuclear uptake of NF- $\kappa$ B.

10 To determine the functional significance of the NLS of p50 and p65 subunits, two peptides were designed and synthesized containing these sequence motifs. The first peptide (referred to as SN50, listed herein as SEQ ID NO:9) contained the signal sequence of K-FGF<sup>16,17</sup> at its amino-terminal region (residues 1-16) and a "functional cargo" in the form of NLS of NF- $\kappa$ B p50 subunit at its carboxy-terminal region (residues  
15 17-26). The second peptide is also a 26-residue peptide (referred to as SN65, listed herein as SEQ ID NO:8) that contains the same hydrophobic sequence and the NLS of p65 subunit. Both peptides were tested for their inhibitory effects on the nuclear translocation of the NF- $\kappa$ B complex in LE-II cells. Inducible  $\kappa$ B binding activity was detectable by electrophoretic mobility shift assay in nuclear extracts from cells treated with LPS for 1 h.<sup>43</sup>  
20 However, this LPS-induced  $\kappa$ B binding activity in nuclear fraction was reduced substantially in the SN50 peptide-treated cells. The inhibition by SN50 peptide was concentration-dependent, reading an 88% inhibition at 50  $\mu$ g/ml. In contrast, no inhibition was observed in SN65 peptide-treated LEII cells. To exclude the possibility that the inhibition was caused by the interference of SN50 peptide in the binding of oligonucleotide probe to the  
25 NF- $\kappa$ B complex, SN50 peptide was incubated *in vitro* with nuclear extracts and radio-labeled probe. This maneuver was without any measurable effect on LPS-induced  $\kappa$ B binding activity, suggesting that inhibition by SN50 peptide resulted from its action at the stage in which the active NF- $\kappa$ B complex moves from cytosol to nucleus. To determine whether the inhibition by SN50 peptide required a hydrophobic, membrane-permeable

sequence, two control peptides (SM and N50 peptides, listed herein as SEQ ID NOS: 1 and 10, respectively) were also tested in the same mobility shift assay. N50 peptide contained the NLS without the hydrophobic sequence, whereas SM peptide contained a hydrophobic sequence without the NLS. Neither of these two peptides showed any significant effect on

5 LPS-induced intracellular translocation of the NF- $\kappa$ B complex from the cytosol to the nucleus. These results suggest that neither the hydrophobic sequence alone (SM peptide) nor the nuclear localization sequence alone (N50 peptide) was sufficient for causing a functional inhibition of the NF- $\kappa$ B. Therefore, the observed inhibitory effect of SN50 must be attributed to its intracellular import, which allowed the interaction of its intrinsic NLS

10 with the nuclear membranes.

SN50 peptide contained the same epitope tag as SM peptide and thus could be recognized by the anti-SM peptide antibody in ELISA. This allowed direct affirmation by an indirect immunofluorescence assay that SN50 peptide was imported into LE-II cells

15 to exert its functional role. Results showed that the intracellular SN50 peptide was distributed in a more nuclear staining pattern as compared to the intracellular SM peptide.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into

20 this application in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be

25 regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

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(ii) TITLE OF INVENTION: A NOVEL METHOD FOR IMPORTING  
BIOLOGICALLY ACTIVE MOLECULES INTO CELLS

(iii) NUMBER OF SEQUENCES: 11

## (iv) COMPUTER READABLE FORM:

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(B) REGISTRATION NUMBER: 33,438  
(C) REFERENCE/DOCKET NUMBER: 2200.0211

## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 404/688-0770  
(B) TELEFAX: 404/688-9880

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

## (ix) FEATURE:

(A) NAME/KEY: Peptide  
(B) LOCATION: 1..16  
(D) OTHER INFORMATION: /label= a  
/note= "Signal peptide amino acid sequence of  
K-FGF"

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## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 17..19
- (D) OTHER INFORMATION: /label= b  
/note= "Spacer region"

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 20..26
- (D) OTHER INFORMATION: /label= c  
/note= "Epitope tag"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala	Ala	Val	Ala	Leu	Leu	Pro	Ala	Val	Leu	Leu	Ala	Leu	Leu	Ala	Pro
1				5					10					15	
Ala	Ala	Ala	Asp	Gln	Asn	Gln	Leu	Met	Pro						
			20					25							

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..7
- (D) OTHER INFORMATION: /label= a  
/note= "Nuclear localization sequence of aFGF"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asn	Tyr	Lys	Lys	Pro	Lys	Leu
1				5		

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..16
- (D) OTHER INFORMATION: /label= a  
/note= "Signal peptide amino acid sequence of K-FGF"

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## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 17..19
- (D) OTHER INFORMATION: /label= b  
/note= "Spacer region"

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 20..26
- (D) OTHER INFORMATION: /label= c  
/note= "Nuclear localization sequence of aFGF"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro  
1 5 10 15

Ala Ala Ala Asn Tyr Lys Lys Pro Lys Leu  
20 25

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..16
- (D) OTHER INFORMATION: /label= a  
/note= "Signal peptide amino acid sequence of  
K-FGF"

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 17..19
- (D) OTHER INFORMATION: /label= b  
/note= "Spacer region"

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 20..26
- (D) OTHER INFORMATION: /label= c  
/note= "Nuclear localization sequence of aFGF"

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 26..28
- (D) OTHER INFORMATION: /label= d  
/note= "Epitope tag"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro  
 1 5 10 15

Ala Ala Ala Asn Tyr Lys Lys Pro Lys Leu Met Pro  
 20 25

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..16
- (D) OTHER INFORMATION: /label= a  
/note= "Signal peptide of K-FGF"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro  
 1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro  
 1 5 10 15

Glu Ile Leu Leu Pro Asn Asn Tyr Asn Ala Tyr Glu Ser Tyr Lys Tyr  
 20 25 30

Pro Gly Met Phe Ile Ala Leu Ser Lys  
 35 40

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu	Ile	Leu	Leu	Pro	Asn	Asn	Tyr	Asn	Ala	Tyr	Glu	Ser	Tyr	Lys	Tyr
1				5					10					15	
Pro	Gly	Met	Phe	Ile	Ala	Leu	Ser	Lys							
				20				25							

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala	Ala	Val	Ala	Leu	Leu	Pro	Ala	Val	Leu	Leu	Ala	Leu	Leu	Ala	Pro
1				5					10					15	
Ile	Glu	Glu	Lys	Arg	Lys	Arg	Thr	Tyr	Glu						
				20				25							

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala	Ala	Val	Ala	Leu	Leu	Pro	Ala	Val	Leu	Leu	Ala	Leu	Leu	Ala	Pro
1				5					10					15	
Val	Asn	Arg	Lys	Arg	Asn	Lys	Leu	Met	Pro						
				20				25							

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val	Asn	Arg	Lys	Arg	Asn	Lys	Leu	Met	Pro
1				5					10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ile	Glu	Glu	Lys	Arg	Lys	Arg	Thr	Tyr	Glu
1				5					10

What is claimed is:

1. A method of importing a biologically active molecule into a cell *ex vivo* comprising administering to the cell a complex comprising the molecule linked to an importation competent signal peptide, thereby importing the molecule into the cell.
2. The method of Claim 1, wherein the administration is performed at about 18°C to about 42°C.
3. The method of Claim 1, wherein the molecule is selected from the group consisting of a peptide, polypeptide, and protein.
4. The method of Claim 1, wherein the molecule is selected from the group consisting of a nucleic acid, carbohydrate, lipid, glycolipid and therapeutic agent.
5. The method of Claim 1, wherein the signal peptide comprises the amino acid sequence set forth in SEQ ID NO:5.
6. A method of importing a biologically active molecule into a cell in a subject comprising administering to the subject a complex comprising the molecule linked to an importation competent signal peptide, thereby importing the molecule into the cell of the subject.
7. The method of Claim 6, wherein the molecule is selected from the group consisting of a peptide, polypeptide, and protein.
8. The method of Claim 6, wherein the molecule is selected from the group consisting of a nucleic acid, carbohydrate, lipid, glycolipid and therapeutic agent.

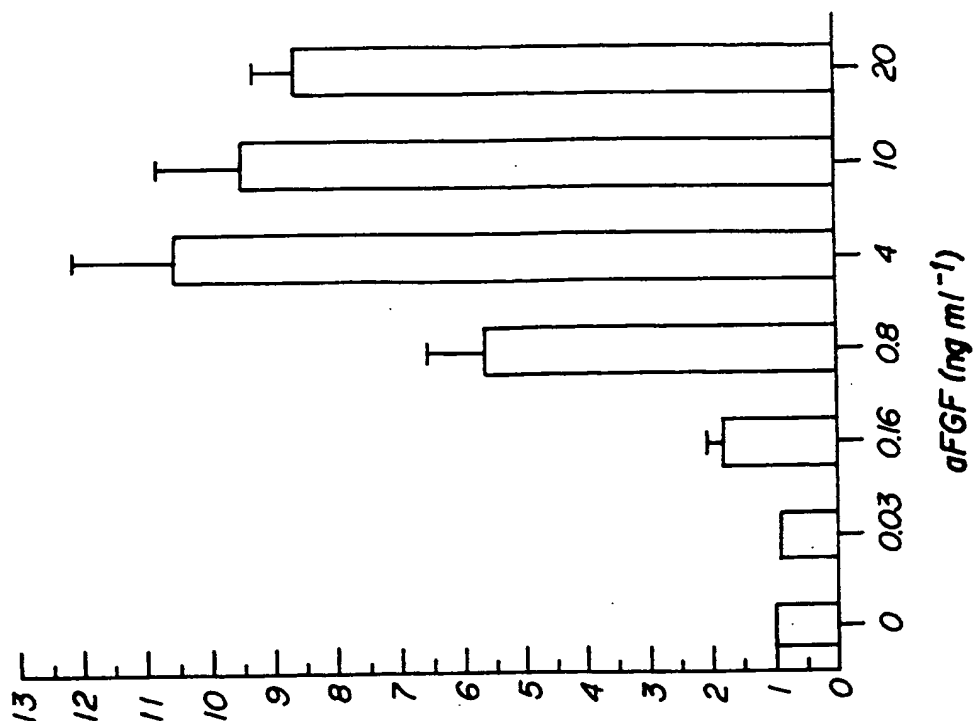


9. The method of Claim 6, wherein the signal peptide comprises the amino acid sequence set forth in SEQ ID NO:5.
10. The method of Claim 6, wherein the molecule is an antigenic peptide.
11. A method of importing a biologically active molecule into the nucleus of a cell in a subject comprising administering to the subject a complex comprising the molecule linked to an importation competent signal peptide and a nuclear localization peptide, thereby importing the molecule into the nucleus of the cell of the subject.
12. The method of Claim 11, wherein the signal peptide comprises the amino acid sequence set forth in SEQ ID NO:5.
13. The method of Claim 11, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO:2.
14. The method of Claim 11, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO:10.
15. The method of Claim 11, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO:11.
16. A method of regulating the growth of a cell in a subject comprising administering to the subject a complex comprising a growth regulatory peptide linked to an importation competent signal peptide, thereby regulating the growth of the cell in the subject.
17. The method of Claim 16, wherein the cell is a tumor cell.

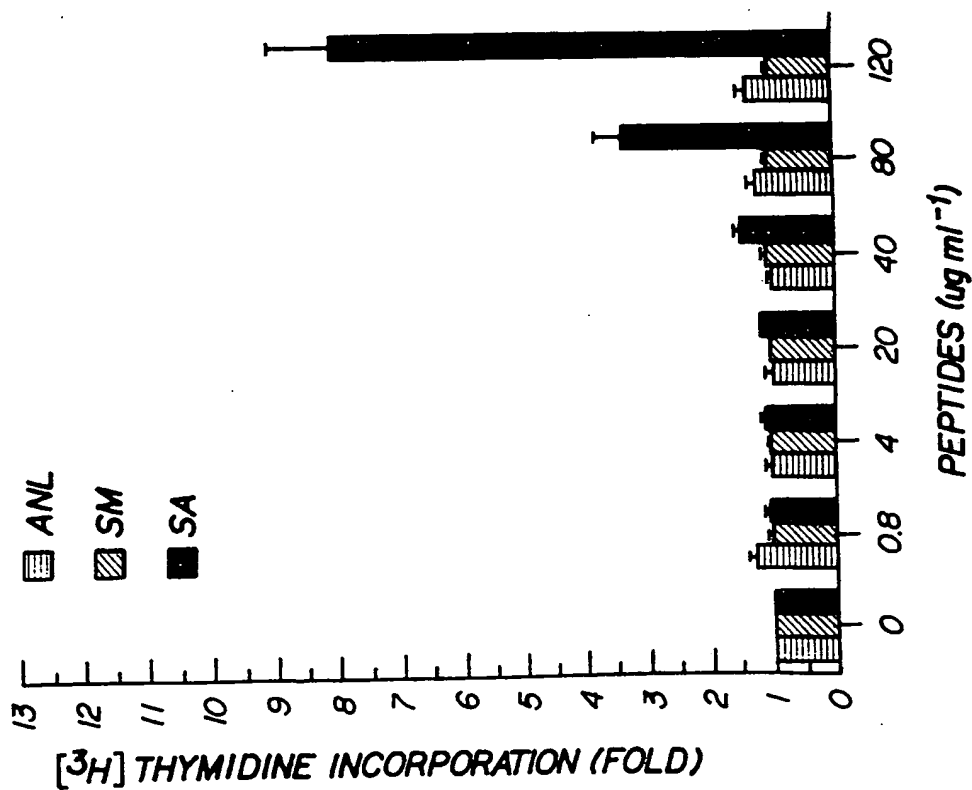
18. The method of Claim 16, wherein the growth regulatory peptide stimulates the cell growth and comprises the nuclear localization sequence of acidic fibroblast growth factor.
19. The method of Claim 18, wherein the growth regulatory peptide comprises the amino acid sequence set forth in SEQ ID NO:3.
20. The method of Claim 18, wherein the growth regulatory peptide comprises the amino acid sequence set forth in SEQ ID NO:4.
21. The method of Claim 16, wherein the growth regulatory peptide inhibits the cell growth.
22. The method of Claim 21, wherein the growth regulatory peptide comprises the amino acid sequence set forth in SEQ ID NO:9.
23. A method of inhibiting expression in a cell in a subject of a gene controlled by transcription factor NF- $\kappa$ B comprising administering to the subject a complex comprising an importation competent signal peptide linked to a nuclear localization peptide of an active subunit of NF- $\kappa$ B complex.
24. The method of Claim 23, wherein the subunit of NF- $\kappa$ B is subunit p50.
25. The method of Claim 24, wherein the complex comprises the amino acid sequence set forth in SEQ ID NO:9.
26. A method of stimulating the immune system of a subject comprising administering to the subject a complex comprising an importation competent signal peptide linked to an antigenic peptide.

27. A complex comprising a biologically active molecule linked to an importation competent signal peptide and to a nuclear localization peptide.
28. The complex of Claim 27, wherein the signal peptide comprises the amino acid sequence set forth in SEQ ID NO 5.
29. The complex of Claim 27, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO 2.
30. The complex of Claim 27, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO 10.
31. The complex of Claim 27, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO 11.
32. A complex comprising an importation competent signal peptide linked to a biologically active molecule selected from the group consisting of a nucleic acid, a carbohydrate, a lipid, a glycolipid and a therapeutic agent.
33. A method of screening signal peptides for the ability to effect the importation of a biologically active molecule into a cell comprising administering to the cell a complex comprising the molecule linked to the signal peptide and determining whether the molecule is imported into the cell, the presence of importation of the molecule indicating a signal peptide which can effect importation.

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**FIG 1B**



**FIG 1A**

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/07539**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 31/00, 38/00, 38/18, 39/00, 45/00; C07K 14/475, 14/50

US CL : 435/320.1; 435/300; 436/501; 514/1, 2

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1; 435/300; 436/501; 514/1, 2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, CAPLUS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ----- Y,P	US, A, 5,324,655 (KRIEGLER ET AL) 28 JUNE 1994, see entire document.	1-4, 6-8, 10, 11, 16-17, 26- 27, 32-33 ----- 5, 9, 12-15, 18- 20, 22-25, 28- 31
A	US, A, 4,935,352 (KOICHI ET AL) 19 JUNE 1990.	1-33
A	WO, A, 93/18185 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 16 SEPTEMBER 1993.	1-33
A	EP, A, 0,225,701 (TAKEDA CHEMICAL INDUSTRIES, LTD.) 16 JUNE 1987.	1-33

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"G" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

06 SEPTEMBER 1995

Date of mailing of the international search report

04 OCT 1995

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## INTERNATIONAL SEARCH REPORT

 International application No.  
 PCT/US95/07539

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES U.S.A., Vol. 87, issued July 1990, Yayon et al, "Autocrine transformation by chimeric signal peptide-basic fibroblast growth factor: Reversal by suramin", pages 5346-5350, see entire document.	1-4, 11, 16-17, 21, 26-27, 32-33 ----- 5, 9, 12-15, 18-20, 22-25, 28-31
X -- Y	METHODS IN ENZYMOLOGY, Vol. 198, issued 1991, Rogelj et al, "Construction and Expression of Transforming Gene Resulting from Fusion of Basic Fibroblast Growth Factor Gene with Signal Peptide Sequence", pages 117-125, see entire document.	1-4, 11, 16-17, 21, 26-27, 32-33 ----- 5, 9, 12-15, 18-20, 22-25, 28-31
A	ANTICANCER RESEARCH, Vol. 10, issued 1990, Egan et al, "Growth Factor Modulation of Metastatic Lung Colonization", pages 1341-1346.	1-33
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES U.S.A., Vol. 84, issued October 1987, Yoshida et al, "Genomic sequence of hst, a transforming gene encoding a protein homologous to fibroblast growth factors and the int-2-encoded protein", pages 7305-7309, see entire document.	5, 9, 12-13 18-20, 22, 25, 28-29
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES U.S.A., Vol. 84, issued May 1987, Taira et al, "cDNA sequence of human transforming gene hst and identification of the coding sequence required for transforming activity", pages 2980-2984, see entire document.	5, 9, 12-13, 18-20, 22, 25, 28-29
Y	CELL, Vol. 50, issued 28 August 1987, Delli Bovi et al, "An Oncogene Isolated by Transfection of Kaposi's Sarcoma DNA Encodes a Growth Factor That Is a Member of the FGF Family", pages 729-737, see entire document.	5, 9, 12-13, 18-20, 22, 25, 28-29
Y	ONCOGENE, Vol. 6, issued 1991, Wang et al, "Cloning and sequence analysis of the human acidic fibroblast growth factor gene and its preservation in leukemia patients", pages 1521-1529, see entire document.	5, 9, 12-13, 18-20, 22, 25, 28-29
Y	JOURNAL OF CELLULAR BIOCHEMISTRY, Vol. 43, issued 1990, Hall et al, "Characterization of the Hamster DDT-1 Cell aFGF/HGBF-1 Gene and cDNA and Its Modulation by Steroids", pages 17-26, see entire document.	5, 9, 12-13, 18-20, 22, 25, 28-29
Y	BIOCHEMICAL AND BIOPHYSICAL COMMUNICATIONS, Vol. 164, No. 3, issued 15 November 1989, Mergia et al, "Structural analysis of the gene for human acidic fibroblast growth factor", pages 1121-1129, see entire document.	5, 9, 12-13, 18-20, 22, 25, 28-29

## INTERNATIONAL SEARCH REPORT

 International application No.  
 PCT/US95/07539

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF VIROLOGY, Vol. 66, No. 6, issued June 1992, Capobianco et al, "p105, the NF-KB p50 Precursor Protein, Is One of the Cellular Proteins Complexed with the v-Rel Oncoprotein in Transformed Chicken Spleen Cells", pages 3758-3767, see entire document.	14, 23-24, 30
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES U.S.A., Vol. 88, issued February 1991, Meyer et al, "Cloning of the DNA-binding subunit of human nuclear factor kB: The level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor alpha", pages 966-970, see entire document.	14, 23-24, 30
Y	CELL, Vol. 62, issued 07 September 1990, Kiernan et al, "The DNA Binding Subunit of NF-kB is identical to Factor KBF1 and Homologous to the rel Oncogene Product", pages 1007-1018, see entire document.	14, 23-24, 30
Y	CELL, Vol. 62, issued 07 September 1990, Ghosh et al, "Cloning of the p50 DNA Binding Subunit of NF-kB: Homology to rel and dorsal", pages 1019-1029, see entire document.	14, 23-24, 30
Y	SCIENCE, Vol. 251, issued 22 March 1991, "Isolation of a rel-Related Human cDNA That Potentially Encodes the 65-kD Subunit of NF-kB", pages 1490-1493, see entire document.	15, 23, 31
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES U.S.A., Vol. 91, issued January 1994, Ray et al, "Physical association and functional antagonism between the p65 subunit of transcription factor NF-kB and the glucocorticoid receptor", pages 752-756, see entire document.	15, 23, 31
Y	CELL, Vol. 64, issued 08 March 1991, Nolan et al, "DNA Binding and Ikb Inhibition of the Cloned p65 Subunit of NF-kB, a rel-Related Polypeptide", pages 961-969, see entire document.	15, 23, 31